

# Dietary cholesterol, rather than liver steatosis, leads to hepatic inflammation in hyperlipidemic mouse models of nonalcoholic steatohepatitis

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# Dietary Cholesterol, Rather than Liver Steatosis, Leads to Hepatic Inflammation in Hyperlipidemic Mouse Models of Nonalcoholic Steatohepatitis

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Nonalcoholic steatohepatitis (NASH) involves liver lipid accumulation (steatosis) combined with hepatic inflammation. The transition towards hepatic inflammation represents a key step in pathogenesis, because it will set the stage for further liver damage, culminating in hepatic fibrosis, cirrhosis, and liver cancer. The actual risk factors that drive hepatic inflammation during the progression to NASH remain largely unknown. The role of steatosis and dietary cholesterol in the etiology of diet-induced NASH was investigated using hyperlipidemic mouse models fed a Western diet. Livers of male and female hyperlipidemic (low-density lipoprotein receptor-deficient [*ldlr*<sup>-/-</sup>] and apolipoprotein E2 knock-in [APOE2ki]) mouse models were compared with livers of normolipidemic wild-type (WT) C57BL/6J mice after short-term feeding with a high-fat diet with cholesterol (HFC) and without cholesterol. Whereas WT mice displayed only steatosis after a short-term HFC diet, female *ldlr*<sup>-/-</sup> and APOE2ki mice showed steatosis with severe inflammation characterized by infiltration of macrophages and increased nuclear factor  $\kappa$ B (NF- $\kappa$ B) signaling. Remarkably, male *ldlr*<sup>-/-</sup> and APOE2ki mice developed severe hepatic inflammation in the absence of steatosis after 7 days on an HFC diet compared with WT animals. An HFC diet induced bloated, “foamy” Kupffer cells in male and female *ldlr*<sup>-/-</sup> and APOE2ki mice. Hepatic inflammation was found to be linked to increased plasma very low-density lipoprotein (VLDL) cholesterol levels. Omitting cholesterol from the HFC diet lowered plasma VLDL cholesterol and prevented the development of inflammation and hepatic foam cells. **Conclusion:** These findings indicate that dietary cholesterol, possibly in the form of modified plasma lipoproteins, is an important risk factor for the progression to hepatic inflammation in diet-induced NASH. (HEPATOLOGY 2008;48:474-486.)

Nonalcoholic fatty liver disease (NAFLD) is a condition ranging from benign lipid accumulation in the liver (steatosis) to steatosis combined with inflammation. The latter is referred to as nonalcoholic steatohepatitis (NASH). NAFLD may be consid-

ered the hepatic event in the metabolic syndrome and is therefore linked with common metabolic syndrome risk factors such as obesity, insulin resistance, hypertension, and dyslipidemia.<sup>1</sup> The prevalence of NAFLD in the general population is increasing, but only a small proportion

Abbreviations: APOE2ki, apolipoprotein E2 knock-in; HE, hematoxylin-eosin; HFC, high-fat cholesterol; HFNC, high fat no cholesterol; KC, Kupffer cell; LDL, low-density lipoprotein; *ldlr*<sup>-/-</sup>, low-density lipoprotein receptor-deficient; MCD, methionine choline-deficient; Mcp1, monocyte chemoattractant protein 1; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NF- $\kappa$ B, nuclear factor  $\kappa$ B; PCR, polymerase chain reaction; TC, total cholesterol; TG, triglyceride; TNF, tumor necrosis factor; WT, wild-type.

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will develop NASH. Estimates in the United States are that only 2%-3% of all adults have NASH, compared with an estimation of 20% of Americans with NALFD.<sup>2</sup>

Steatosis alone is considered a relatively benign and reversible condition. The transition toward NASH represents a key step in pathogenesis, because it will set the stage for further damage to the liver including fibrosis, cirrhosis, and liver cancer. The actual risk factors that drive hepatic inflammation during the progression to NASH remain largely unknown. Therefore, knowledge about events that induce hepatic inflammation is of great importance for the diagnosis and treatment of NASH.

Currently, NASH is thought to develop via the "two-hit" model.<sup>3</sup> According to this hypothesis, hepatic steatosis is the critical first hit and prerequisite for further liver injury. A second hit can be represented by oxidative stress.<sup>4</sup> However, recent reports have raised doubts about steatosis as a prerequisite for development of inflammation during NASH progression.<sup>5-7</sup> In line with these observations, we have shown previously that in apolipoprotein E2 knock-in (APOE2ki) mice, a mouse model with a human-like lipoprotein profile, hepatic steatosis and inflammation (that is, steatohepatitis) develop very rapidly when fed a Western diet with moderate amounts of fat. After just 2 days of a Western diet enriched with triglycerides and cholesterol, female APOE2ki mice show marked liver inflammation<sup>8</sup> that developed alongside rather than subsequent to steatosis.

The APOE2ki mouse carries the defective human APOE2 isoform that replaces the endogenous mouse apoe gene. Apolipoprotein E is highly expressed in macrophages and has been shown to influence several inflammatory processes.<sup>9</sup> Therefore, to exclude defects in apolipoprotein E as the cause of such early inflammation in the liver, we first investigated diet-induced NASH development in another hyperlipidemic mouse model that does not have a defect in apolipoprotein E: the low-density lipoprotein receptor-deficient (*ldlr*<sup>-/-</sup>) mouse.<sup>10</sup> C57BL6/J wild-type (WT) mice, with the genetic background of both mouse models, were used as a control. Second, pilot experiments revealed a difference between female and male *ldlr*<sup>-/-</sup> and APOE2ki mice, because the male mice did not develop steatosis after short-term high-fat feeding. Consequently, we investigated male hyperlipidemic APOE2ki and *ldlr*<sup>-/-</sup> mice to determine whether steatosis is necessary for hepatic inflammation to develop. Third, based on the results of these diet intervention studies, a correlation was found between plasma total cholesterol (TC) and hepatic inflammation. Therefore, the role of plasma TC was further investigated.

## Materials and Methods

Extended Materials and Methods can be found online, Shortly:

In the first experiment, female C57BL6/J and *ldlr*<sup>-/-</sup> mice were fed chow or high-fat diet with cholesterol (HFC) (21% milk butter, 0.2% cholesterol) for 2, 4, 7, and 21 days. In the second experiment, male C57BL6/J, *ldlr*<sup>-/-</sup>, and APOE2ki mice were fed either an HFC diet for 7 days or were kept on chow. In the third experiment, male and female C57BL6/J, *ldlr*<sup>-/-</sup>, and APOE2ki mice were fed an HFC diet or a high-fat diet without cholesterol (HFNC) for 7 days. Collection of specimens, lipid analysis, RNA isolation, complementary DNA synthesis, and quantitative polymerase chain reaction (PCR) were performed as described.<sup>8</sup>

**Taqman Low Density Arrays.** Taqman Low Density Arrays 96a, containing 4 × 96 annotated and validated individual TaqMan Gene Expression Assays (Supplementary Table 1), were performed. Per individual assay, 2 ng complementary DNA of a single liver was loaded together with TaqMan Universal PCR Master Mix. Each group consisted of five mice. Data were normalized to *Ppia* expression.

**Liver Histology.** Four-micrometer paraffin-embedded liver sections were stained with hematoxylin-eosin (HE) and periodic acid-Schiff–diastase. Frozen liver sections (7 μm) were fixed in acetone and stained with CD68 (FA11) or Mac1 (M1/70). Pictures were taken with a Nikon DMX1200 digital camera and ACT-1 version 2.63 software.

**Electron Microscopy.** Livers were freshly isolated, perfused, and fixed with 2.5% glutaraldehyde. Tissue fragments were postfixed in 1% osmium tetroxide, dehydrated, and embedded in epoxy resin. Sections were cut for light microscopy (toluidine blue) and electron microscopy. Electron microscopy sections were analyzed on a Philips CM100 TEM.

**Statistical Analysis.** Data were analyzed using Graphpad Prism 4.0. Groups were compared using two-tailed nonpaired *t* tests or analysis of variance with a Dunnett posttest based on the statistical relevance. Data are expressed as the mean ± standard error of the mean and were considered significant at *P* < 0.05.

## Results

**Plasma Lipid Levels in Female Hyperlipidemic Mouse Models.** Female *ldlr*<sup>-/-</sup>, APOE2ki, and WT mice were fed HFC diet for up to 3 weeks, and changes in plasma lipids were monitored (experiment 1). Results on female APOE2ki mice have been published previously<sup>8</sup> and are included in Fig. 1 (black bars) for clarity. WT mice displayed only minor changes in their lipid profile. Compared with control chow levels, female *ldlr*<sup>-/-</sup> mice showed increased plasma triglyceride (TG) (Fig. 1A) and

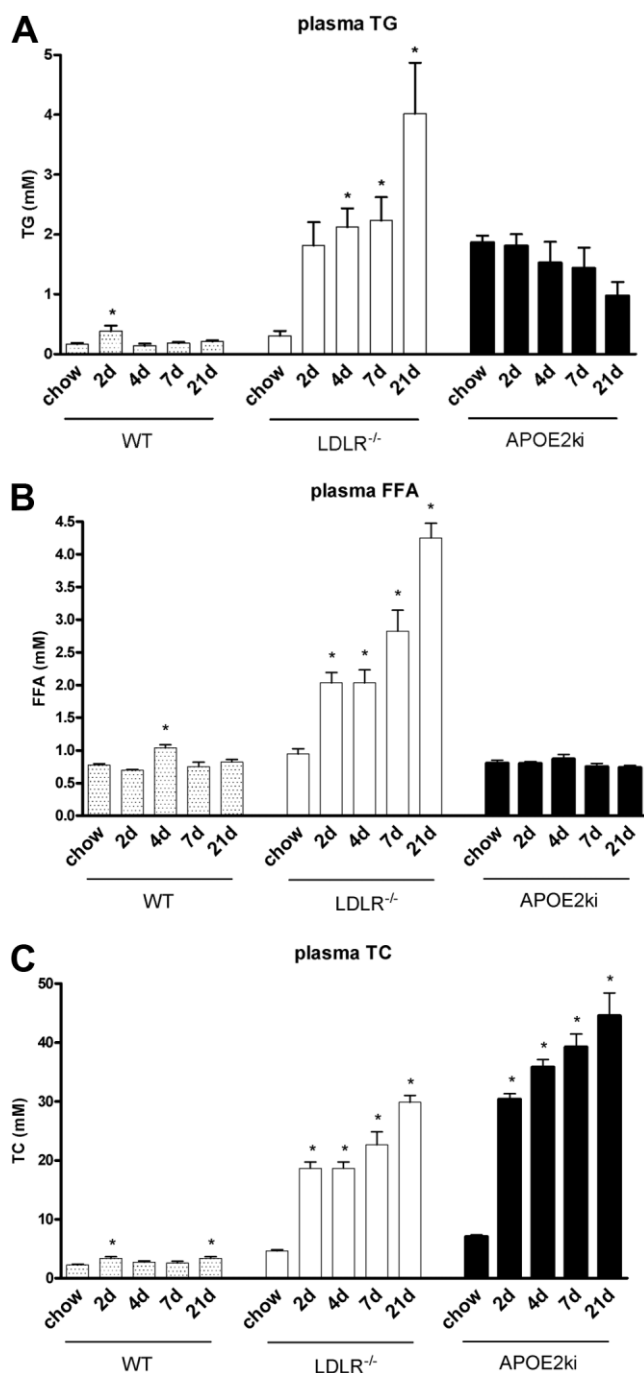


Fig. 1. Plasma lipid levels of three female mouse models at several time points after HFC treatment: 2, 4, 7, and 21 days compared with chow-fed control mice. (A) Plasma triglycerides (TG). (B) Plasma free fatty acids (FFA) and (C) plasma total cholesterol (TC). Bars represent time points (2, 4, 7, and 21 days) and are grouped per genotype: WT mice (grey bars), *ldlr*<sup>-/-</sup> mice (white bars), and APOE2ki mice (black bars). Statistical analysis was performed using one-way analysis of variance with Dunnett's posttest. \*Significantly different from chow diet levels.

free fatty acid (Fig. 1B) levels and diet-induced hypercholesterolemia (Fig. 1C) upon HFC feeding. Historical data of female APOE2ki mice (black bars) show that these animals have elevated basal plasma TG levels that tended

to decrease with time (Fig. 1A), whereas free fatty acids remained at basal levels (Fig. 1B) and total cholesterol (TC) levels increased markedly throughout the dietary period (Fig. 1C).<sup>8</sup>

**Only Female *ldlr*<sup>-/-</sup> and APOE2ki Mice Develop Liver Inflammation.** APOE2ki and *ldlr*<sup>-/-</sup> mice showed equal accumulation of lipid droplets in their livers after 7 days of an HFC diet, which was comparable with livers of control WT animals (Fig. 2A). Fig. 2B shows that all mouse models had similar increases of liver TG after short periods of HFC feeding (Fig. 2B). Liver TC showed a similar response (Fig. 2C). HFC feeding thus induced a degree of hepatic steatosis in *ldlr*<sup>-/-</sup> and APOE2ki mice, similar to what was observed in WT animals. These observations were confirmed by oil Red O staining (data not shown).

Because HE staining revealed inflammatory clusters (Fig. 2A) in the livers, antibodies against the macrophage marker Mac1 were used to identify inflammatory cells. The number of Mac1-positive cells was counted to determine the level of liver inflammation. Interestingly, WT livers were completely free of inflammation upon HFC feeding, whereas *ldlr*<sup>-/-</sup> mice displayed inflammatory cell clusters similar to those observed previously in APOE2ki mice<sup>8</sup> (Fig. 2D), albeit that the inflammation was less severe in *ldlr*<sup>-/-</sup> mice.

Gene expression analysis of several inflammatory genes was performed in livers of the mouse models outlined at 2, 4, 7, and 21 days after HFC feeding. These genes were monocyte chemoattractant protein 1 (Mcp1) (Fig. 2E), CD68 (Fig. 2F), a macrophage marker, and tumor necrosis factor (TNF) (Fig. 2G), a cytokine. Expression of these genes in *ldlr*<sup>-/-</sup> and APOE2ki mice was strongly up-regulated after HFC feeding. In contrast, control WT mice showed only moderate increases in liver gene expression, apparently insufficient to drive an overt inflammatory response as determined by liver histology.

Hence, in HFC-induced fatty liver, female APOE2ki and *ldlr*<sup>-/-</sup>, but not WT mice, are sensitive to developing inflammation, indicating that diet-induced steatosis does not necessarily lead to the immediate development of an inflammatory response in the liver.

#### **Liver Gene Expression Profiling Reveals an Inflammatory Profile in *ldlr*<sup>-/-</sup> but not in WT Female Mice.**

To investigate the hepatic response to an HFC diet in more detail, custom Taqman Low Density Array assays were designed to compare the expression of 96 genes involved either in lipid transport and metabolism or inflammation. The expression of Mcp1, CD68, and TNF, determined via quantitative PCR, was similar between *ldlr*<sup>-/-</sup> and APOE2ki mice (Fig. 2E-G). Additionally, previous microarray analysis has already shown a pro-



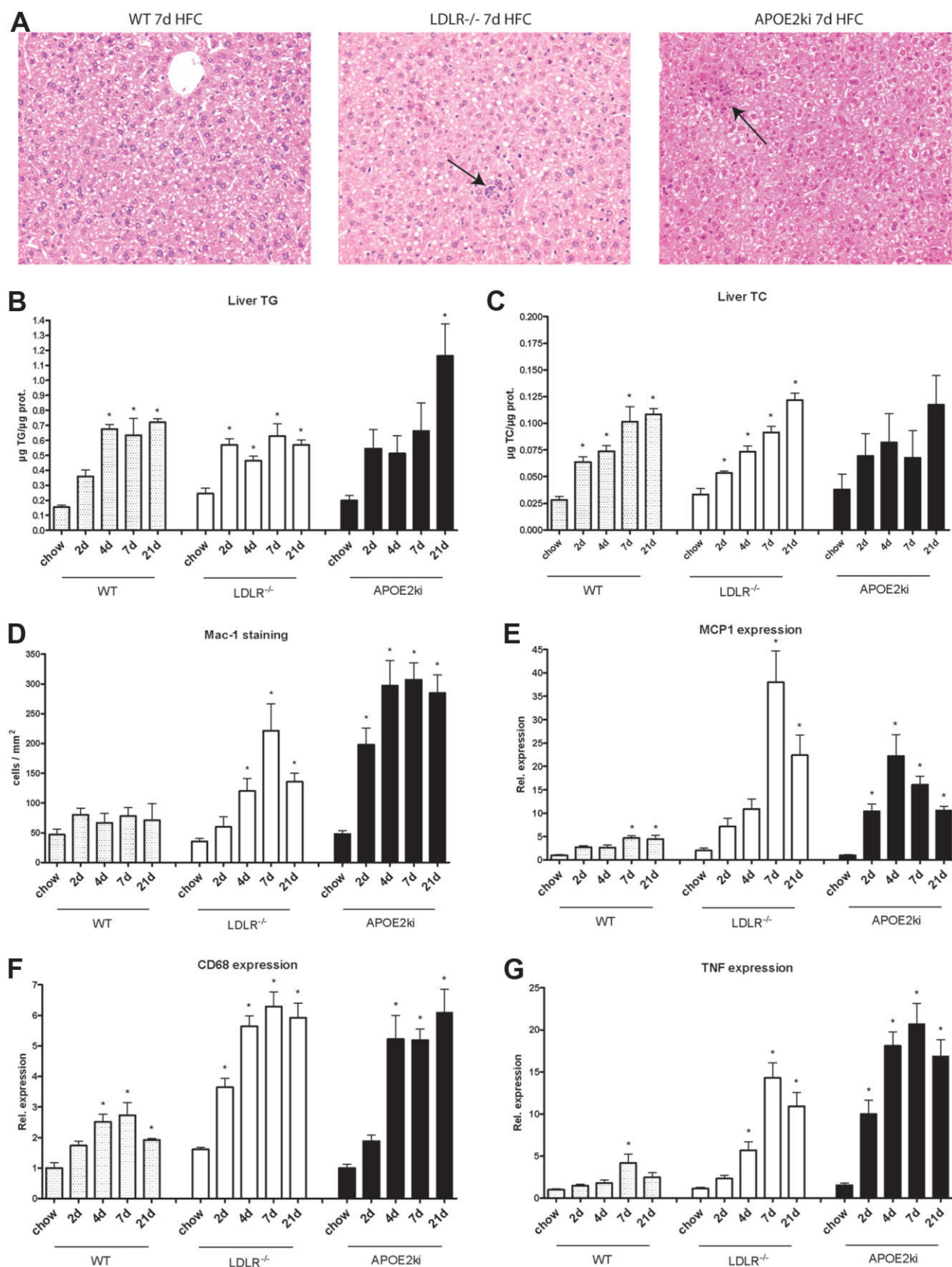


Fig. 2. NASH parameters in female mice. (A) Representative pictures (magnification  $\times 200$ ) of HE-stained liver sections were taken of female mice after 7 days on an HFC diet. Arrows indicate inflammatory cell clusters. Liver (B) TG and (C) TC levels were quantified biochemically. (D) Liver sections were stained for Mac1 (CD11b) and counted. Gene expression analysis with quantitative reverse-transcription PCR for three known inflammatory marker genes: (E) monocyte chemoattractant protein 1, (F) CD68, and (G) TNF. Data were set relative to WT animals on a chow diet. Bars represent time points (2, 4, 7, and 21 days) and are grouped per genotype: WT mice (grey bars), *ldlr*<sup>-/-</sup> mice (white bars), and APOE2ki mice (black bars). Statistical analysis was performed using one-way analysis of variance with Dunnet's posttest. \*Significantly different from chow diet levels.

**Table 1. Liver Gene Expression: Genes Involved in Lipid Metabolism**

Gene	Group	WT	LDLR <sup>-/-</sup>	Gene	Group	WT	LDLR <sup>-/-</sup>
		HFC Versus Chow	HFC Versus Chow			HFC Versus Chow	HFC Versus Chow
Cd36	Lipid uptake	1.4	1.0	Fasn	Lipid metabolism synthesis	0.5	0.4
Fabp1	Lipid uptake	1.2	0.9	LXRa	Lipid metabolism synthesis	1.6	1.1
Lipc	Lipid uptake	1.1	0.9	FXR	Lipid metabolism synthesis	1.1	0.7
Lpl	Lipid uptake	1.3	3.7*	Scd1	Lipid metabolism synthesis	1.2	0.6
Lrp1	Lipid uptake	1.5*	1.1	Srebf1	Lipid metabolism synthesis	1.2	1.3
Slc27a1	Lipid uptake	1.1	0.9	Abca1	Cholesterol efflux	1.5	1.7*
Acaa1a	Lipid oxidation and efflux	1.3	1.0	Cyp7a1	Cholesterol efflux	2.0	1.6
Acox1	Lipid oxidation and efflux	1.2	0.8	Scarb1	Cholesterol efflux	1.8	1.4*
Cpt1a	Lipid oxidation and efflux	1.5	0.8	Adfp	Intracellular lipid distribution	1.3	0.8
Crot	Lipid oxidation and efflux	1.5	0.9	Cav1	Intracellular lipid distribution	1.2	0.9
Ech1	Lipid oxidation and efflux	0.9	0.8	Cav2	Intracellular lipid distribution	1.2	0.9
Hadha	Lipid oxidation and efflux	1.1	1.0	M6prbp1	Intracellular lipid distribution	1.4	1.0
Mttp	Lipid oxidation and efflux	1.0	0.8*	Npc1	Intracellular lipid distribution	1.0	0.6
Ppara	Lipid oxidation and efflux	1.4	0.9	Idi1	Cholesterol metabolism	0.2*	0.1*
Ppard	Lipid oxidation and efflux	1.2	1.2	Insig2	Cholesterol metabolism	1.5	0.7
Pparg	Lipid oxidation and efflux	1.1	1.1	Cyp8b1	Other	1.3	0.3

Table shows gene abbreviation and classification according to function. Expression is shown as fold change compared with levels of animals on standard chow diet for WT and *ldlr*<sup>-/-</sup> female mice after 7 days of an HFC diet. Values marked with an asterisk (\*) indicate significant changes (Student t test) compared with chow.

found inflammatory response after feeding female APOE2ki mice an HFC diet.<sup>8</sup> The response of *ldlr*<sup>-/-</sup> mice to 7 days of HFC feeding was compared with that of WT mice. Tables 1 and 2 show changes in expression levels for these genes and indicate the pathway to which each gene belongs. Genes involved in lipid metabolism showed only few changes after 7 days of HFC diet in either mouse model (Table 1). Inflammatory gene expression, including several known targets of NF- $\kappa$ B, was markedly up-regulated in female *ldlr*<sup>-/-</sup> mice, but not in female WT mice (Table 2). The inflammatory response consisted mainly of the increased expression of macrophage-specific genes (Table 2), such as CD68, Fc Gamma receptor 1, and Mac1, but not other immune cell-specific genes such as CD19 (B cells), CD4 (T helper cells), CD8a (cytotoxic T cells), and myeloperoxidase (neutrophils). These data suggest that inflammation is mainly related to macrophage accumulation and activation. Additionally, *Icam1* and *Vcam1*, both of which are involved in inflammatory cell migration and invasion, were up-regulated. Additionally, genes involved in chemotaxis (Table 2), such as *ccl2* (*mcp1*), *ccl3*, and *ptgs2* were strongly regulated, indicating an important role of these gene products in the development of hepatic inflammation.

**Steatosis Is not Necessary for the Development of HFC Diet-Induced Hepatic Inflammation.** Male *ldlr*<sup>-/-</sup> and APOE2ki mice were put either on chow or an HFC diet for 7 days (experiment 2). Male WT animals were used as a control. Both male *ldlr*<sup>-/-</sup> and APOE2ki mice showed increased diet-induced plasma TG (Fig. 3A) and TC (Fig. 3B) compared with control mice, although

APOE2ki mice were less responsive than *ldlr*<sup>-/-</sup> mice. The changes in TG were similar to those observed in female *ldlr*<sup>-/-</sup> mice, whereas APOE2ki mice differed in their TG response, because female APOE2ki mice had elevated starting levels of TG that did not change after dietary intervention (Fig. 1A). Whereas the female APOE2ki mice were more responsive with respect to plasma TC (Fig. 1C), male APOE2ki mice had a lower increase than *ldlr*<sup>-/-</sup> mice.

Biochemical assessment of liver lipids showed no increase in liver TG levels in male hyperlipidemic mice. Male WT mice did show an increase in hepatic TG; however, the levels after 7 days of an HFC diet did not exceed the ones displayed in male *ldlr*<sup>-/-</sup> and APOE2ki mice (Fig. 3C). On the other hand, liver TC levels did rise significantly after an HFC diet (Fig. 3D) in all models. Likewise, oil Red O staining did not reveal overt steatosis (data not shown).

Lipid accumulation is generally considered an initial and causal factor in the progression from steatosis to NASH.<sup>11</sup> Surprisingly, despite the lack of steatosis in male mice, there was a severe inflammatory response, reflected by a three-fold to five-fold increase in Mac1-positive cells (Fig. 3E) and increased gene expression of *Mcp1* (Fig. 3F), CD68 (Fig. 3G), and TNF (Fig. 3H) compared with controls. These responses were more pronounced in APOE2ki than in *ldlr*<sup>-/-</sup> male mice. Overall, this indicates that liver lipid accumulation is not a prerequisite for hepatic inflammation to develop in these mouse models.

**Foam Cells and Modified Lipoproteins in Hyperlipidemic Mice.** Previously, we found that female APOE2ki mice displayed an increase in size of CD68-

**Table 2. Liver Gene Expression: Genes Involved in Inflammation**

Gene	Group	WT HFC Versus Chow	LDLR <sup>-/-</sup> HFC Versus Chow	Gene	Group	WT HFC Versus Chow	LDLR <sup>-/-</sup> HFC Versus Chow
Cd19	Cell markers	0.8	1.4	Socs3	Anti-inflammatory	1.3	1.9
Cd4	Cell markers	2.0	1.3	Cd14	General inflammation	1.8	4.5
Cd68	Cell markers	1.7	3.1*‡	Cd40	General inflammation	2.1	2.7*
Cd8a	Cell markers	1.4	2.0	Cd80	General inflammation	2.7	2.7*
Fcgr1	Cell markers	2.2	2.4*‡	Cd86	General inflammation	2.2	3.0*
Itgam/Mac1	Cell markers	1.5	5.7*‡	CsfF	General inflammation	0.6	0.9
Mpo	Cell markers	3.0	7.9	Cxcl10	General inflammation	3.0	3.5*
Icam1	Cell markers	1.9	3.0	Ifng	General inflammation	1.1	1.7
Vcam1	Cell markers	1.0	3.0	Il18	General inflammation	1.0	0.9
Vegfa	Cell markers	1.1	1.0	Il18r1	General inflammation	1.9	4.0*
Ccl2/mcp1	Chemotaxis	3.0	9.5*†	Il1b	General inflammation	1.4	6.3*
Ccl3/mip1	Chemotaxis	3.4	11.2*†	Il1r1	General inflammation	0.8	2.0*
Ccr2	Chemotaxis	1.8	1.9*†	Il1r2	General inflammation	1.1	5.4
Ptgs2/Cox2	Chemotaxis	1.2	25.4*†	Il6	General inflammation	1.6	6.0
Cat	Oxidative stress	1.1	0.8	Il6ra	General inflammation	0.6	0.7
Gsta2	Oxidative stress	0.5*	0.2*	Nfkbia	General inflammation	1.2	1.4
Hmox1	Oxidative stress	1.8	2.8*	Saa1	General inflammation	2.3*	19.9*
Ikbkb	Oxidative stress	1.0	1.3	Stat1	General inflammation	1.8	1.8*
Por	Oxidative stress	1.3	0.8	Stat3	General inflammation	1.2	1.4
Bcl2	Apoptosis	1.2	2.0*	Tlr2	General inflammation	2.3*	4.9*
FasI	Apoptosis	2.0	2.2*	Tlr4	General inflammation	1.5	2.4*
Il10	Anti-inflammatory	2.5	1.7	Tnf	General inflammation	3.0	10.0*
Il10ra	Anti-inflammatory	1.5	2.9*	Tnfrsf1a	General inflammation	1.3	1.0
Socs1	Anti-inflammatory	2.6	2.3	Tnfrsf1b	General inflammation	1.3	1.4

Table shows gene abbreviation and classification according to function. Expression is shown as fold change compared with levels of animals on standard chow diet for WT and *ldlr*<sup>-/-</sup> female mice after 7 days of an HFC diet. Values marked with an asterisk (\*) indicate significant changes (Student t test) compared with chow.

†Regulated genes involved in chemotaxis.

‡Regulated genes used as cell markers.

positive cells after dietary intervention.<sup>8</sup> Immunostaining against CD68 now indicated a comparable increase in size rather than number of CD68-positive cells (that is, Kupffer cells and macrophages) in livers of male and female *ldlr*<sup>-/-</sup> and APOE2ki mice, but not of WT mice (Fig. 4A) (experiments 1 and 2). Additionally, toluidine blue staining clearly illustrated that the cells with a foamy appearance are located in the sinusoidal space of the liver, suggesting that they are Kupffer cells (KCs) (Fig. 4B). Further detail was provided with electron microscopy (Fig. 4C). Electron microscopy pictures showed clear differences between livers of the animals. KCs appeared to have more cytoplasm and filled a larger fraction of the sinus, indicating that these cells are swollen compared with chow-fed animals. In HFC-fed animals, the cytoplasm of KCs contained lipid droplets and filled lysosomes. Moreover, these cells contained cholesterol crystals, which is indicative of an uptake of cholesterol by these cells.

**Omitting Dietary Cholesterol Reduces Plasma Very Low-Density Lipoprotein TC and Protects Against Developing Hepatic Inflammation.** A consistent finding was that plasma TC was increased in the mouse models that developed hepatic inflammation. Accordingly, we hypothesized that plasma TC is an important determi-

nant of hepatic inflammation. To test this hypothesis, mice (both male and female) were put on the HFC and HFNC diet (experiment 3). Omitting dietary cholesterol-induced lower levels of very low-density lipoprotein (VLDL)-TC than the HFC diet in both sexes of the hyperlipidemic mice (Fig. 5) lowered total plasma TC by approximately 50% in APOE2ki and *ldlr*<sup>-/-</sup> animals (data not shown). Female WT mice also displayed lowering of TC levels, whereas this was not observed in male WT mice (data not shown).

Omission of dietary cholesterol did result in lower TG and TC levels in WT controls but did not diminish liver TG content in APOE2ki and *ldlr*<sup>-/-</sup> mice (Fig. 6A,B). In male mice, omitting cholesterol tended to enhance TG content in the livers (Fig. 6C). The level of liver TC remained low in female APOE2ki mice and in male *ldlr*<sup>-/-</sup> mice (Fig. 6 B,D).

Strikingly, Mac1 staining of liver sections showed that macrophage infiltration was limited when *ldlr*<sup>-/-</sup> and APOE2ki mice of both sexes were put on the HFNC diet (Fig. 6 E,F). Control mice did not show hepatic inflammation in any of the conditions. HE staining revealed that upon feeding male and female *ldlr*<sup>-/-</sup> and APOE2Ki mice an HFNC diet, no swollen, foamy KCs were detected

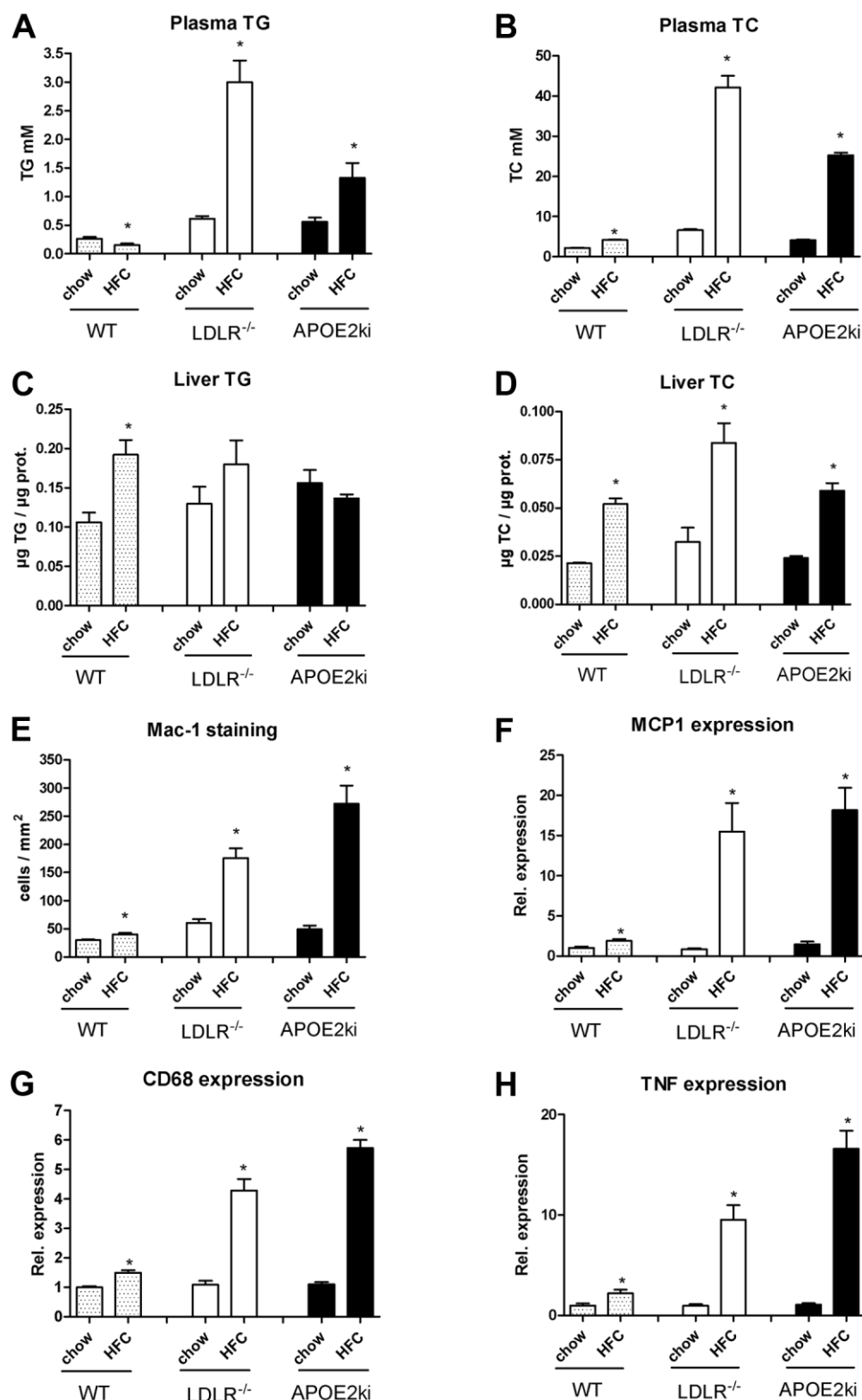


Fig. 3. Plasma and liver lipid levels in male mice. (A) Plasma TG and (B) Plasma TC were determined for male mice. Data are from animals fed a chow diet or 7 days of an HFC diet. (C) Liver TG and (D) liver TC cholesterol levels were quantified biochemically. (E) Liver sections were stained with antibodies against Mac1 (CD11b) and counted. Gene expression analysis with quantitative reverse-transcription PCR for three known inflammatory genes: (F) monocyte chemoattractant protein 1, (G) CD68, and (H) TNF. Data was set relative to WT animals on a chow diet. Bars are grouped per genotype: WT mice (grey bars), *ldlr*<sup>-/-</sup> mice (white bars), and APOE2ki mice (black bars). Statistical analysis was performed using Student *t* tests. \*Significantly different from chow diet levels.



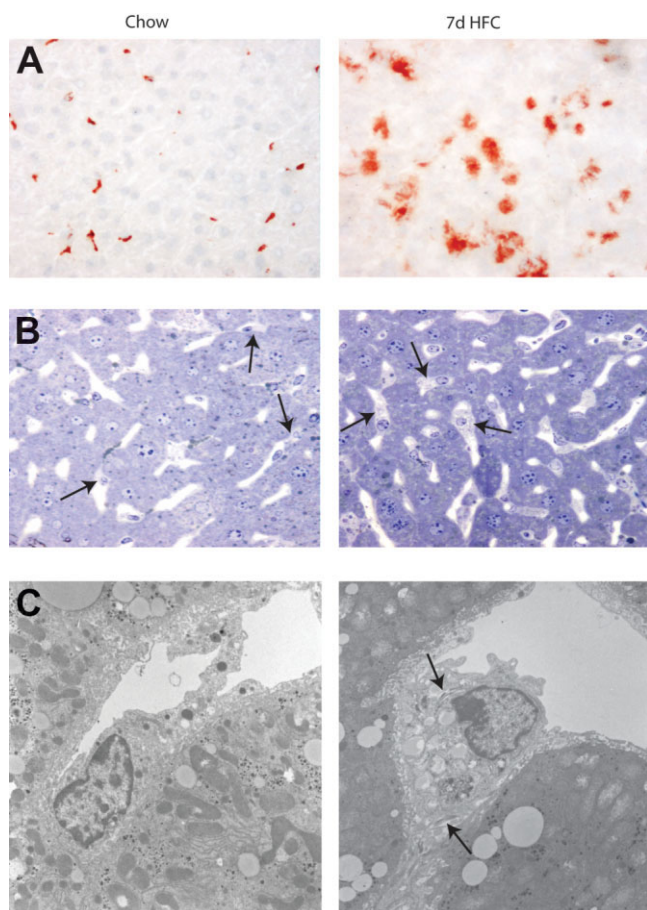


Fig. 4. Hepatic foam cells. (A) Representative pictures (magnification  $\times 400$ ) of female APOE2ki liver sections stained against CD68 on a control chow diet and after 7 days of an HFC diet. (B) Representative pictures (magnification  $\times 400$ ) of toluidine-stained liver sections from APOE2ki female mice on a chow diet and after 7 days of an HFC diet. Arrows indicate foamy KCs. (C) Representative electron microscopy photographs (magnification  $\times 1550$ ) of female APOE2ki mice fed either a chow diet or an HFC diet. Arrows indicate cholesterol crystals.

(Fig. 7). Additional evidence was found with periodic acid-Schiff–diastase staining. Supplementary Fig. 1 shows the lack of swollen KCs in HFNC-fed mice compared with HFC-fed mice.

Accordingly, expression levels of *Mcp1*, CD68, and TNF were down-regulated in female mice fed an HFNC diet compared with those fed an HFC diet, and similar trends were observed in male mice (Supplementary Fig. 2). Thus, lower levels of diet-induced VLDL-TC are associated with less hepatic inflammation in *ldlr*<sup>-/-</sup> and APOE2ki mice.

## Discussion

This study clearly dissociates steatosis and inflammation in the livers of hyperlipidemic mice. High-fat feeding induced a very early inflammatory response in the livers of

*ldlr*<sup>-/-</sup> and APOE2ki female mice. In contrast, female WT mice developed comparable steatosis but no inflammation. Additionally, we found that in male mice, inflammation developed rapidly in the absence of steatosis. Together, these results suggest that liver inflammation can develop independently of steatosis upon high-fat feeding. Subsequent experiments showed that omitting cholesterol from the HFC diet prevented VLDL-TC accumulation and hepatic inflammation, while parameters of steatosis remained largely unaffected. The presence of bloated foamy KCs only in HFC-fed hyperlipidemic mice suggests that scavenging of modified lipoproteins by KCs may initiate this early inflammation.

**Hyperlipidemic Mice Are Sensitive to Develop Early Diet-Induced NASH.** Until now, the best characterized and most known models for NASH are mice deficient for leptin (Ob/Ob) or mice fed a diet deficient in methionine and choline (MCD).<sup>11</sup> However, Ob/Ob mice do not spontaneously develop liver inflammation but require a second hit, like the administration of lipopolysaccharide to activate inflammatory signaling. Furthermore, mutation of the leptin gene is not common in human obese NAFLD patients. MCD-fed mice display all the hallmarks of NASH, from steatosis to inflammation and fibrosis development. However, MCD-fed animals tend to lose weight and display lowered plasma triglyceride (TG) levels<sup>11</sup> and are therefore very different from human NASH patients, who are mostly obese and/or hyperlipidemic.

Dyslipidemia is commonly associated with NAFLD. It has been postulated that abnormalities in lipid metabolism—such as the increase of serum TG, TC, and low-density lipoprotein-TC levels and decrease of high-density lipoprotein-TC levels—may be contributing factors of NASH development.<sup>12</sup> Consequently, hyperlipidemic mice have been shown to develop diet-induced NASH, not only in APOE2ki mice<sup>8</sup> but also in *ldlr*<sup>-/-</sup> and apolipoprotein E-deficient (*apoe*<sup>-/-</sup>) mice.<sup>13</sup> Unlike human subjects, WT mice carry most of their lipids in high-density lipoproteins. In contrast, *ldlr*<sup>-/-</sup> mice and mice with various defects in apolipoprotein E have a human-like lipoprotein profile and may serve as physiological mouse models to study the early progression of NASH.

Medium-scale gene expression analysis showed that, despite the presence of steatosis, not many lipid genes were regulated in either *ldlr*<sup>-/-</sup> or in WT control mice. This is probably due to the early time point of 7 days of high-fat feeding, which might be too early to evoke a large transcriptional response of these genes. In line with this, we have found that HFC-induced expression of genes involved in lipid metabolism increases gradually with

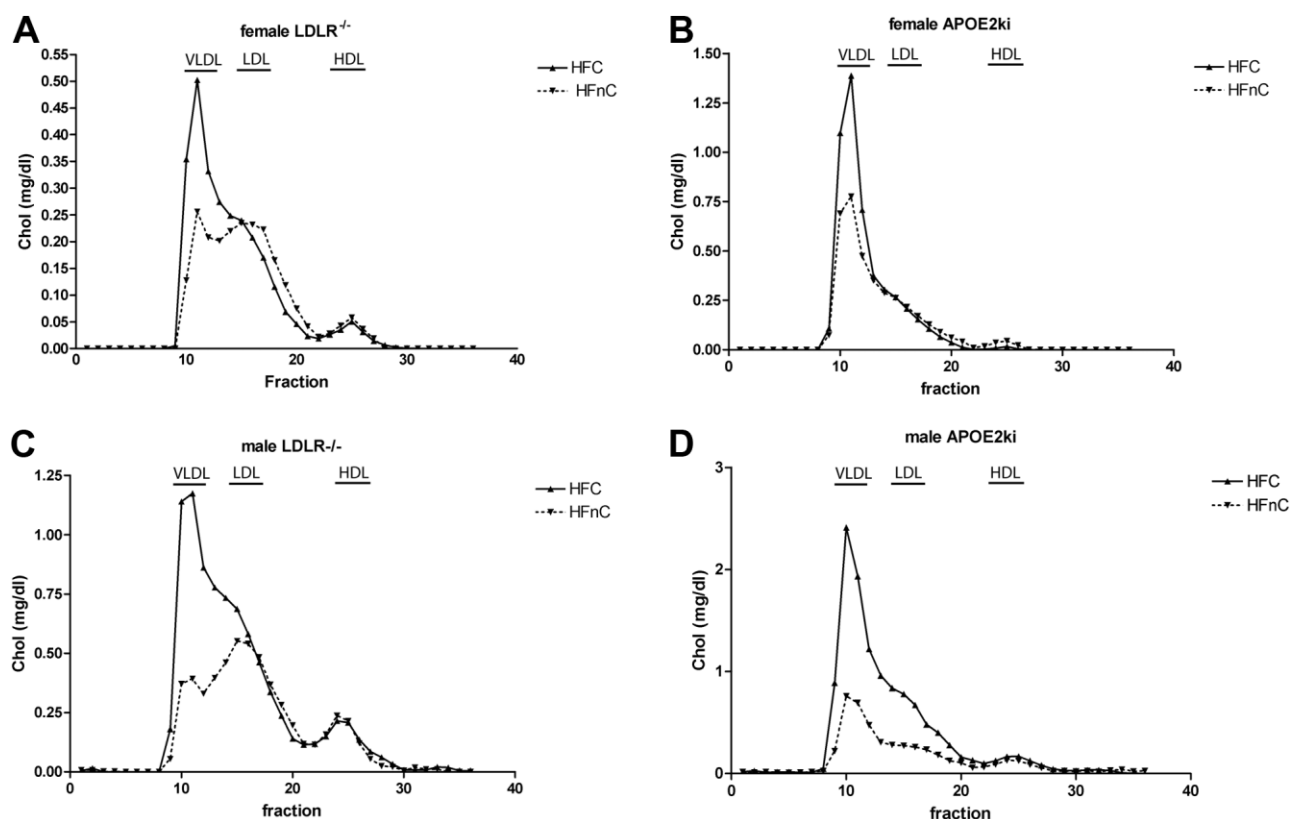


Fig. 5. Treatment with an HFNC diet lowers VLDL cholesterol levels. Shown are lipoprotein fractions after 7 days of an HFC diet and 7 days of an HFNC diet in (A) female *ldlr*<sup>-/-</sup> mice, (B) female APOE2ki mice, (C) male *ldlr*<sup>-/-</sup> mice, and (D) male APOE2ki mice.

time.<sup>8</sup> Alternatively, the physiological response on lipids may also be due to secondary feedback mechanisms regulated on the protein level rather than the gene level, resulting in the lack of transcriptional regulation.

In contrast to the lipid genes, a large set of inflammatory genes was regulated in the HFC-fed *ldlr*<sup>-/-</sup> mice, but not in WT mice, confirming our histological data. The data show that the inflammatory response consisted mainly of genes involved in chemotaxis and infiltration of macrophages. Furthermore, many genes that were up-regulated are known targets of the transcription factor NF- $\kappa$ B, suggesting an important role for this transcription factor in response to HFC feeding and NASH development, as has been postulated in HFC-fed APOE2ki mice<sup>8</sup> and in MCD-fed mice with a C57BL6/J background.<sup>14</sup> Moreover, in NASH patients, NF- $\kappa$ B expression was found to be up-regulated and correlated with hepatic inflammation and fibrosis.<sup>15</sup> The other regulated genes are involved in several inflammatory signaling pathways, such as interleukin-18, interleukin-1, interleukin-6, TNF, and Toll-like receptor signaling.

**Steatosis Is Dissociated from the Development of Hepatic Inflammation.** In contrast to the two-hit model, where hepatic steatosis is generally considered as the first hit in the transition toward inflammation,<sup>1</sup> male

mice had an inflamed liver even without steatosis. The lack of steatosis development in male mice compared with female mice was somewhat surprising, because estrogen is normally known to be protective against NASH. It has been shown that estrogen replacement in estrogen-deficient mice lowers steatosis development.<sup>16</sup> We postulate that the lack of steatosis in male mice may be explained by the fact that in the livers of male mice, peroxisome proliferator-activated receptor isoforms are up to 100-fold more active than in livers of female mice.<sup>17</sup> Therefore, it is conceivable that in male mice, activation of peroxisome proliferator-activated receptor can compensate for the increased lipid load. In support of this, we showed previously that feeding female APOE2ki mice an HFC diet for short periods did not activate peroxisome proliferator-activated receptor  $\alpha$  significantly.<sup>8</sup>

Another gender-specific difference was found in APOE2ki mice, as female APOE2ki mice displayed no change in plasma TG levels, while male APOE2ki mice did show increases upon HFC intake. A possible explanation for this observation may be that estrogens can increase activities of hepatic lipase and lipoprotein lipase,<sup>18</sup> which could lead to increased hydrolysis of TG in the plasma.

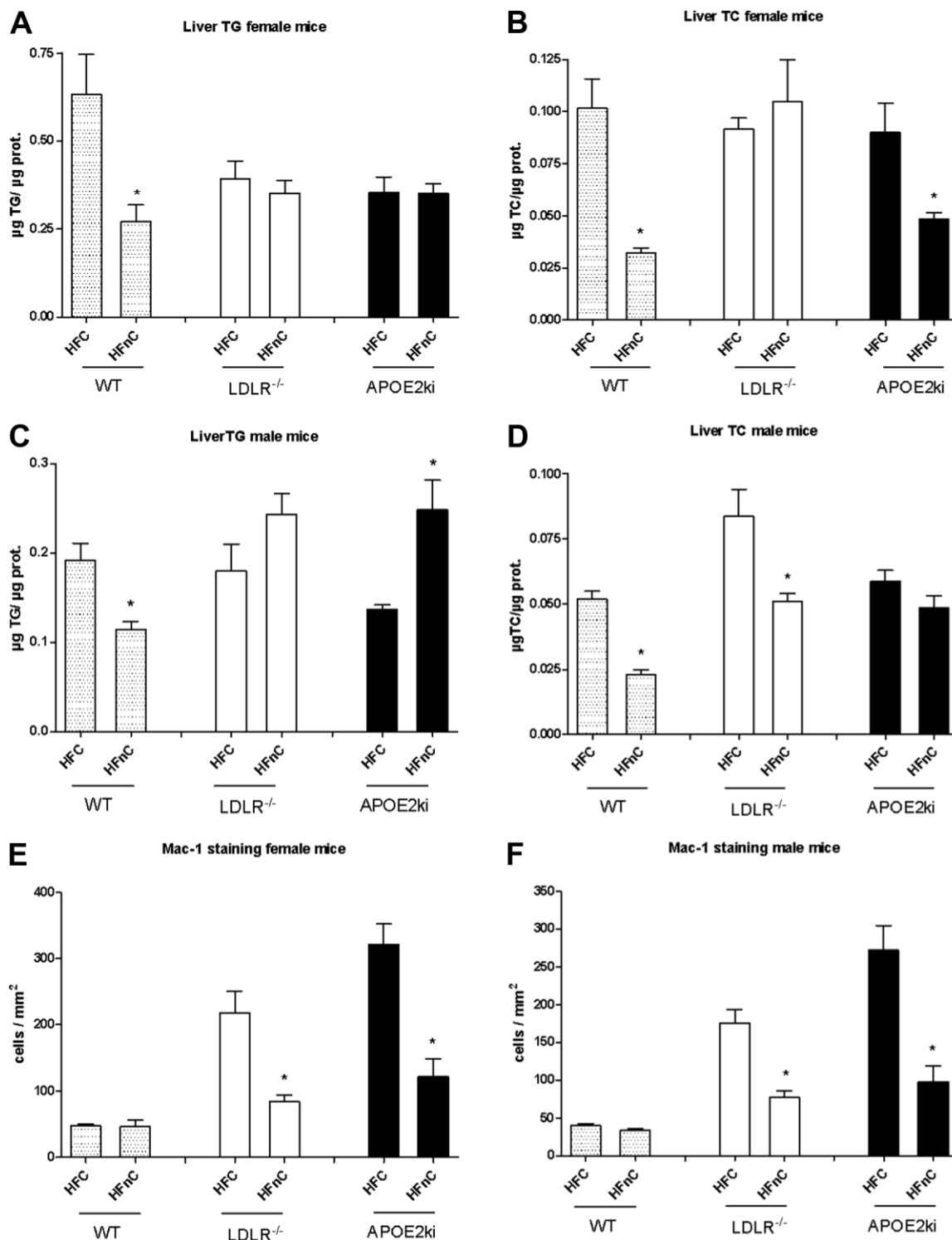


Fig. 6. Omitting cholesterol from the high-fat diet prevents inflammation development without affecting steatosis. Biochemical measurements of liver lipids are shown for TG in (A) female and (C) male mice and for TC content in (B) female and (D) male mice after 7 days of treatment with an HFC or HFNC diet. Counting of Mac1-positive cells is shown in liver sections of (E) female and (F) male mice. Data are shown from WT mice (grey bars), *ldlr*<sup>-/-</sup> mice (white bars), and APOE2ki mice (black bars). Statistical analysis was performed using Student *t* tests. \*Significantly different from HFC diet levels.



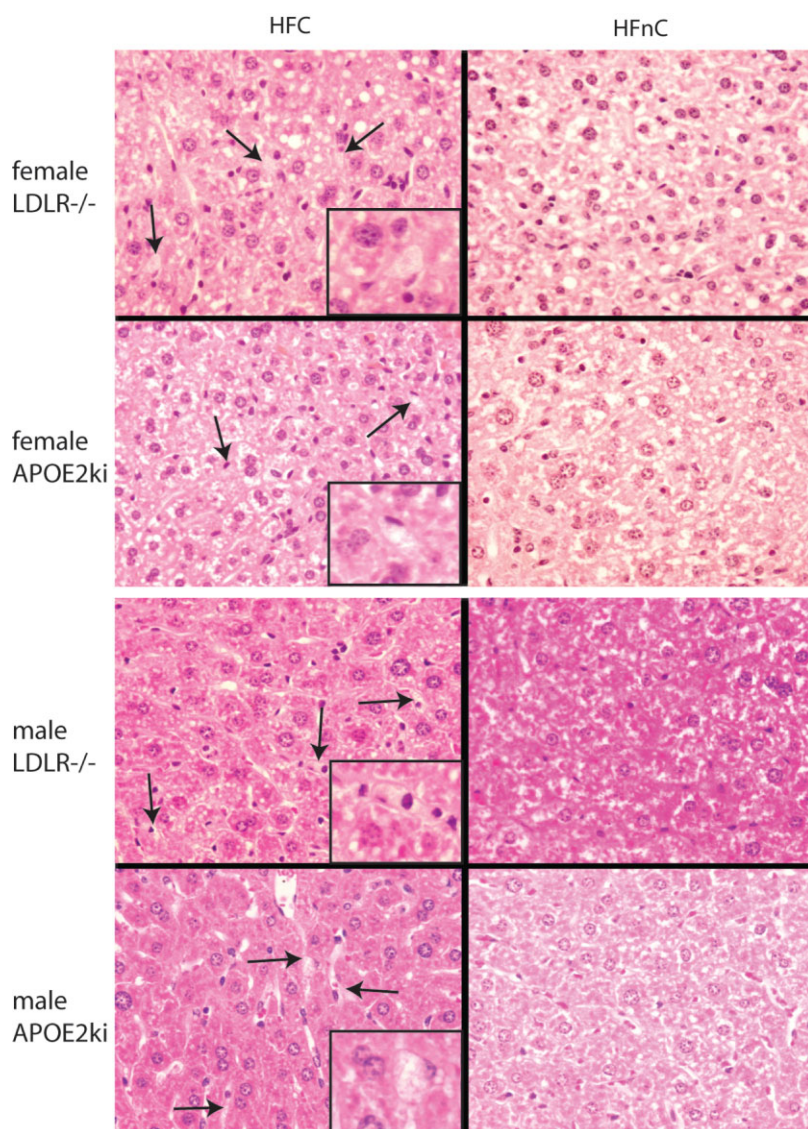


Fig. 7. An HFnC diet does not induce hepatic foam cells. Representative pictures (magnification  $\times 400$ ) of HE-stained liver sections from *Idlr*<sup>-/-</sup> and *APOE2ki* male and female mice after 7 days on an HFC and HFnC diet. Arrows indicate foamy KCs. Inserts show foamy KCs when present on higher magnification.

Clearly, steatosis is not mandatory for progression toward hepatic inflammation in the mouse models used. In fact, others have postulated TG accumulation in the liver may even serve as a protective mechanism against inflammation development, by acting as a reservoir for harmful free fatty acids.<sup>6</sup>

**Plasma Cholesterol May Mediate Hepatic Inflammation.** We found a correlation between plasma TC and the development of hepatic inflammation, rather than with steatosis. This observation led us to hypothesize plasma TC as an important cause for the development of inflammation in these animals. Feeding the animals an HFnC diet kept plasma VLDL-TC low and prevented the development of inflammation in all hyperlipidemic mouse models without diminishing liver TG. Liver TG even increased in male *APOE2ki* mice without any apparent explanation, an observation that further strengthens the dissociation of steatosis from hepatic inflammation.

Recent publications also point to the importance of dietary cholesterol in liver inflammation and NASH both in rodents<sup>5,19</sup> and rabbits.<sup>20</sup> Moreover, in human subjects, it was found that high-cholesterol feeding increased C-reactive protein and serum amyloid A levels.<sup>21</sup>

Recently, Mari et al.<sup>5</sup> reported that high-cholesterol feeding may serve as the first hit that sensitizes rat livers to develop hepatic inflammation after exposure to a second hit, like TNF or FAS. In contrast, we observed that high-cholesterol feeding alone is sufficient to cause a very early inflammatory response. Several differences between the studies could explain this dissimilarity. First, Mari et al. used rats, which are known to be more resistant to developing hyperlipidemia than mice.<sup>22</sup> Our study incorporated mouse models with genetic modifications specifically involved in plasma lipoprotein clearance and lipid metabolism. Consequently, it is possible that other mechanisms may be of greater importance in a rat model.



Second, the manner of inducing steatosis was different. We used a mild high-fat diet, whereas Mari et al. used choline deficiency to evoke steatosis in their rats. Moreover, their diet contained high levels of cholesterol (2%) supplemented with sodium cholate, but no elevated TG content. In these concentrations, both cholesterol and cholate have been shown to induce an inflammatory response in the livers of C57BL/6/J mice.<sup>23</sup> The diet used in our study contained elevated TG levels to evoke steatosis and had cholesterol levels of only 0.2%, which is much closer to the average daily cholesterol intake in humans. Therefore, the diet used in our study appears to be more relevant in terms of induction of NASH in human patients.

Interestingly, in line with the present observations, male *apoe*<sup>-/-</sup> mice were shown to develop liver inflammation when fed cholesterol levels of 0.25% and higher.<sup>24</sup> This again points to an important role for dietary cholesterol in hepatic inflammation. However, this inflammatory response was investigated after several weeks of a high-fat diet and in the presence of steatosis. Our data suggest a very early impact of dietary cholesterol—possibly in the form of plasma VLDL-TG—on hepatic inflammation, regardless of steatosis development.

**KCs May Initiate Early Hepatic Inflammation by Scavenging Modified Lipoproteins.** The mice used in our study are commonly used in atherosclerosis research, because they have atherogenic lipoprotein profiles due to increased modified remnant lipoproteins (oxidized low-density lipoprotein).<sup>25</sup> Oxidized low-density lipoprotein (LDL) binds to the scavenger receptor CD36 and scavenger receptor class A (SRA), which are also present on KCs, and can trigger an inflammatory response.<sup>26,27</sup> In livers of the mice with hepatic inflammation, we found bloated, foamy cells, which resemble lipid-laden KCs, as has been described.<sup>13,28</sup>

Injection of modified LDL has been shown to result in an immediate uptake preferentially by nonparenchymal cells such as KCs.<sup>29,30</sup> Moreover, it has been shown that modified LDL injection in mice activates the hepatic NF- $\kappa$ B pathway and subsequent inflammation.<sup>31</sup> Therefore, it is possible that in our hyperlipidemic mouse models, circulating levels of modified lipoproteins are scavenged by hepatic KCs, thereby triggering an inflammatory response. On the other hand, in WT mice, cholesterol-rich lipoproteins are rapidly cleared from the blood by hepatocytes via the LDL receptor before they can be modified and taken up by KCs.

Taken together, it is feasible that oxidized LDL may be a causal factor for the development of diet-induced hepatic inflammation. A paper have shown a correlation between postprandial LDL-conjugated dienes and he-

patic necroinflammation and fibrosis development in human subjects.<sup>32</sup> LDL levels, possibly in modified form, were also found to be increased in NASH patients,<sup>12</sup> confirming the clinical relevance of our findings.

In conclusion, this study demonstrates that dietary cholesterol, possibly in the form of modified plasma lipoproteins, rather than liver steatosis, may be a risk factor for NASH development. Currently, most therapies for NASH patients involve weight loss, and most diagnostic tests of NASH severity depend solely on the degree of steatosis. Further studies may unravel the exact contribution of cholesterol to the risk of developing NASH and may provide evidence for alternative strategies for new therapies and markers for diagnostic tests.

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